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**APPLICANT:** RUDOLF KRUMBHOLZ ET AL :

**SERIAL NO:** (To be assigned) : **ART UNIT:**

**FILED:** -HEREWITH- : **EXAMINER:**

**FOR:** "COLUMN-CHROMATOGRAPHIC :  
SEPARATION METHOD" :

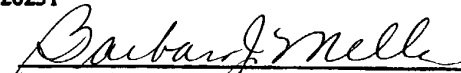
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**TRANSMITTAL OF APPLICATION PAPERS  
TO U.S. DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. §371  
(37 CFR 1.494 OR 1.495)**

This Transmittal Letter is based upon PTO Form 1390 (as revised in May, 1993).

The above-identified applicant(s) (jointly with their assignee) have filed an International Application under the P.C.T. and hereby submit(s) to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay.
4. ☒ A proper Demand for International Preliminary Examination (IPE) was made to the appropriate Authority (IPEA) within the time period required.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2)) --
  - a. ☒ is transmitted herewith (required when not transmitted by International Bureau). See WIPO Publication WO 01/07137.
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A (verified) translation of the International Application into the English language is enclosed.
7. ☐ Amendments to the (specification and) claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
  - e. ☐ will be submitted with the appropriate surcharge.
8. ☐ A translation of the amendments to the claims (and/or the specification) under PCT Article 19 (35 U.S.C. §371(c)(3)) is enclosed or will be submitted with the appropriate surcharge.

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9. ☒ An oath or declaration/power of attorney of the inventor(s) (35 U.S.C. §371[c][4]) will follow.  
[ ] and is attached to the translation of (or a copy of) the International Application.  
[ ] and is attached to the substitute specification.

10. [ ] A translation of at least the Annexes to the IPE Report under PCT Article 36 (35 U.S.C. §371[c][5]) is enclosed.

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98 is enclosed.  
12. ☒ An Assignment for recording and a separate cover sheet in compliance with 37 CFR 3.28 and 3.31 will follow.  
13. [ ] A FIRST preliminary amendment is enclosed.  
A SECOND or SUBSEQUENT preliminary amendment is enclosed.  
14. [ ] A substitute specification (including claims, abstract, drawing) is enclosed.  
15. [ ] A change of power of attorney and/or address letter is enclosed.  
16. ☒ Other items of information:

- ☒ This application is being filed pursuant to 37 CFR 1.494(c) or 1.495(c), and any missing parts will be filed before expiration of--

[ ] 22 months from the priority date under 37 CFR 1.494(c), or

☒ 32 months from the priority date under 37 CFR 1.495(c).

- ☒ The undersigned attorney is authorized by the International applicant and by the inventors to enter the National Phase pursuant to 37 CFR 1.494(c) or 1.495(c).

The following additional information relates to the International Application:

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- ☒ Receiving Office: EPO
- ☒ IPEA (if filing under 37 CFR 1.495): EPO
- ☒ Priority Claim(s) (35 USC §§ 119, 365):  
German Appln. 199 34 168.0 filed -July 21, 1999-.
- ☒ A copy of the International Search Report is
  - ☐ enclosed.
  - ☒ attached to the copy of the International Application.
- ☒ A copy of the Receiving Office Request Form is enclosed.
- ☒ Form PCT/IB/308 (2) sheets
- ☒ Amended Sheets pages -20- in English and  
page -21- in German  
claims 1 through 8.

The fee calculation is set forth on the next page of this Transmittal Letter.

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FEE CALCULATION SHEET

[ ] A check in payment of the filing fee, calculated as follows, is attached (37 CFR 1.492):

Basic Fee ..... \$890.00

Total Number of claims in  
excess of (20) times \$18 ..... -0-

Number of independent claims  
in excess of (3) times \$94 ..... -0-

Fee for multiple dependent  
claims \$280 ..... -0-

TOTAL FILING FEE... \$890.00

Kindly send us the official filing receipt.

[X] The Commissioner is hereby authorized to charge this fee and any additional fees which may be required or to credit any overpayment to Deposit Account No. 03-2775. This is a "general authorization" under 37 CFR 1.25(b), except that no automatic debit of the issue fee upon allowance is authorized. An additional copy of this page is attached.

Respectfully submitted,

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WEM:bjm (9600\*2)

Enclosures

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COLUMN-CHROMATOGRAPHIC  
SEPARATION  
METHOD

Rudolf Krumbholz  
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-and-  
Manfred Treitz

ENGLISH TRANSLATION  
OF  
INTERNATIONAL APPLICATION

PCT/EP00/06853 ..... IFD: -July 18, 2000-

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# Description

## Column-chromatographic separation method

- 5 The present invention relates to a column-chromatographic separation method using a liquid and low molecular weight fluid as eluant for the isolation and/or purification and/or preparative recovery of (natural) materials from mixtures, preferably mixtures of natural products.
- 10 Column-chromatographic separation methods are playing an increasing role in raw materials processing, in product purification and in chemical synthesis, both on a laboratory scale and in industrial-scale production. They are likewise indispensable in chemical analysis for the isolation, identification and quantitative/qualitative determination of individual components in mixtures. Further important applications for such separations are in recycling where poisons and pollutants, but also materials of value, are separated from waste/used materials such as wastewater and waste gases.
- 15
- 20 However, for cost reasons, only few column-chromatographic separation methods have been utilized commercially. Particularly for the purposes of preparative isolation of raw materials, in particular from mixtures of natural products, and in analysis thereof, column-chromatographic separations such as HPLC (high performance liquid chromatography) and SFC (super-critical fluid chromatography) are used.
- 25

- HPLC makes use of the observation that the separation performance of a column increases with decreasing particle size of the stationary phase. HPLC employs a considerably finer chromatographic separation material (3-10  $\mu\text{m}$ ) than does gel chromatography (35-75  $\mu\text{m}$ ) or "classical" column chromatography (120-200  $\mu\text{m}$ ). The fineness of the separation materials does, however, require the use of high pressures (up to 40 MPa), which requires special engineering measures. The columns usually have a length of 5-100 cm (but usually up to 25 cm) and an internal diameter of 1-25  $\mu\text{m}$ .
- 30
- 35 When filled with silica gel or similar porous material (10  $\mu\text{m}$  or less), a 25 cm long column can have 5 000 theoretical separation stages - even 65 000 theoretical separation stages/m can be achieved, depending on the stationary phase. Typical HPLC separations are carried out at room

15 SFC has become established as a column-chromatographic separation method using gases ( $\text{CO}_2$ ,  $\text{N}_2\text{O}$ ,  $\text{SF}_6$  etc.) in the supercritical state as mobile phase. In SFC, liquids and gases are heated under pressure and above the critical temperature and critical pressure in each case are converted into the supercritical state. In this state, supercritical fluids are distinguished from true liquids not only by their lower density, much lower viscosity and much higher diffusion coefficient but also, in particular, by their excellent solvent capability. For this reason, supercritical fluids comprising  $\text{CO}_2$ , ethylene, propane, ammonia, dinitrogen dioxide, water, toluene, nitrogen heterocycles, etc., are employed in extraction, known as supercritical fluid extraction (SFE or SCFE; also referred to as "destruction") of natural products. Known applications are, for example, the extraction of caffeine from coffee by means of supercritical  $\text{CO}_2$ , of bitter components of hops, fragrances, of hydrocarbons from petroleum or coal, for the reactivation of heterogeneous catalysts (cf. Wenclawiak (editor), Analysis with Supercritical Fluids: Extraction and Chromatography, Berlin: Springer 1992).

35

Numerous separations at temperatures below the critical temperature of the mobile phase used have been described; cf. K. Anton, C. Berger (Ed.); *Packed Columns*, Marcel Dekker inc., New York, Basel, Hongkong, 1998.



- The separation of mixtures of materials, in particular mixtures of natural products, by means of SFC is described in a series of publications, for example JP-A-10/316,991; Chirality (1992); 4(4), 252-62; J. chromatogr. (1989) 464(1), 125-37; Nihon Yukagakkaishi (1997), 46(11), 1335-1345;
- 5 Semin. Food Anal. (1996), 1(2), 101-116; J. High Resolut. Chromatogr. (1996), 19(10), 569-570; Jasco Rep. (1991) 33(1), 6-11; Anal. Chem. (1990), 62(12), 394R-402R; and J. Chromatogr. Sci. (1990), 28(1), 9-14.
- 10 On page 64 (Anton supra), Vérillon and Coleman describe the temperature and pressure range for working with fluids such as CO<sub>2</sub> as from 3 to 200°C and 90-400 bar. The separations are carried out in the supercritical and subcritical region, but not in the liquid region i.e. in a pressure range below the critical pressure and temperature.
- 15 On page 409 in chapter 14 (supra Anton), Jusforques describes the working range required as being temperatures of from 0 to 150°C and pressures of from 100 to 350 bar (SFC apparatus: Super C12 for preparative SFC).
- 20 The company Prochrom likewise indicates a working pressure range of from 100 to 350 bar at temperatures of from 10 to 80°C (Prochrom, brochure on semipreparative supercritical fluid chromatograph, type: SuperC 20, 1998, Chamigneulle, France).
- 25 EP 0 099 765 B1 discloses that the inlet pressure of a mixture into the column has to be greater than the critical pressure of the eluant, and in the range from 1.05 to 3. Pressures lower than the critical pressure are expressly described as precipitation conditions.
- 30 In NO 163139, M. Perrut describes only the supercritical and subcritical range, namely pressures of 70-250 bar and temperatures of from 25 to 80°C, as separation conditions.
- 35 In the prior art, no chromatographic separations using mobile phases in a working range below the critical temperature and pressure are described.
- Schultz et al. (Schultz, W.G.: Randall, J.M.: Food Technol. 24, 1282 (1970)) describe only an unspecific extraction at 22°C and 63 bar of flavor



In the method of the invention, such lower molecules are present in liquid form as eluant in the mobile phase.

- 5 Preference is given to using those eluants which are known to a person skilled in the art of SFC.

However, liquid carbon dioxide is very particularly preferred and is employed in the liquid temperature range from -57°C to 31.3°C, preferably  
10 from 0 to 20°C.

Furthermore, liquid carbon dioxide is very particularly preferred and is employed in the pressure range above 30 bar, preferably from 30 to 150 bar and very preferably from 30 to 60 bar. The liquid pressure range of  
15 carbon dioxide is from 5.2 to 73.7 bar.

The critical pressure of carbon dioxide is 73.7 bar and its critical temperature is 31.3°C. The triple point of carbon dioxide is at -57°C and 5.2 bar.

- 20 Of course, the eluants mentioned can also be present in admixture.

The working range of the low molecular weight eluants used is the liquid phase at the associated p,T values.

- 25 These p,T values likewise prevail in the chosen chromatographic separation column through which the eluant is passed as mobile phase.

For the purposes of the present invention, a column-chromatographic separation method means that separation of materials occurs by partition,  
30 i.e. by dissolution in two, mutually immiscible phases, and by adsorption on a solid (adsorbent) in a stationary phase in the presence of an eluant as mobile phase. It is thus possible to refer to the method as liquid-solid chromatography (LSC). There are terminology recommendations by IUPAC for the terms utilized here, and these are expressly incorporated by  
35 reference.

In a particular embodiment, the mobile phase comprising an eluant is admixed with a modifier customary in chromatography, for example an

alcohol which is liquid under the operating conditions, e.g. ethanol or methanol. This modifier can, if desired, be admixed with further auxiliaries and additives such as acids or bases (e.g. acetic acid or diethylamine).

- 5 The method of the invention has numerous advantages compared to known methods such as HPLC or SFC methods.

A particular advantage compared to known HPLC or SFC methods is that it is possible to employ lower pressures, for example pressures of only up to  
10 about 70 bar, in the method of the invention. This enables cost savings since all components required, e.g. pumps, pressure sensors or pipes, can be designed accordingly or made smaller. Safety measures required for occupational hygiene and operational safety can be reduced.

- 15 It has surprisingly been found that the method of the invention can be applied in the presence of any commercial solid stationary phases used for the purposes of HPLC and/or SFC.

However, preference is given to columns containing modified or unmodified  
20 phases based on silica gel, aluminum oxide or titanium dioxide or supported polymeric stationary phases e.g. diol phase, aminopropyl phase, RP8 and RP18 phases applied to silica gel, aluminum oxide or titanium dioxide, with particular preference being given to these phases being present on silica gel supports.

25

The method of the invention can be employed for column lengths within a wide range. The columns are usually at least 10 cm long, but preference is given to lengths of 0.25-2.0 m, particularly preferably 1.1-1.7 m.

- 30 These longer columns ensure a high separation performance and can be achieved since the method of the invention causes no large pressure drop in the column.

The pressure drop can be estimated by means of formula 1 in table 1.  
35 Since the viscosity of the liquid eluants used is low, the resulting pressure drop is also small. As a result of the low pressure drop per cm of column, the column can be made correspondingly long at the same particle diameter of the stationary phase or, compared to HPLC, smaller particle

diameters can be used for a given column length.

The method of the invention can be operated using customary apparatuses as are also used in known SFC methods.

5

To explain the invention further, the operation of a customary SFC apparatus on an industrial scale using the eluant CO<sub>2</sub> is described below.

10 CO<sub>2</sub> to be fed in is taken from a liquid CO<sub>2</sub> reservoir and cooled by means of a heat exchanger. The CO<sub>2</sub> conveyed by the pump is brought to supercritical conditions by means of a heat exchanger (T > 31°C, p > 74 bar), typically 40-60°C. This CO<sub>2</sub> flows through the separation column and is depressurized via a pressure regulating valve. This pressure regulating valve regulates the column outward pressure which prevails at  
15 the end of the separation column and is kept above the critical pressure (p > 74 bar). This ensures supercritical separation conditions in the separation column. The CO<sub>2</sub> is cooled as a result of the adiabatic expansion at the pressure regulating valve. The CO<sub>2</sub> is subsequently heated by means of a heat exchanger and brought into the gaseous state.  
20 In a precipitator, the product is separated from the gaseous CO<sub>2</sub>. The product is separated from the gaseous CO<sub>2</sub>. The gaseous CO<sub>2</sub> goes via the heat exchanger, which liquefies it again, to the reservoir. A closed CO<sub>2</sub> circuit is thus obtained. The product is taken discontinuously from the stock container by means of the pump and is injected into the separation column.  
25 For this customary SFC arrangement, a total of at least four heat exchangers are necessary, which is very energy intensive.

In the context of the method of the invention, there are numerous possible functional arrangements in addition to the procedure described above for  
30 the SFC method, and some of these will be described below.

The particular advantage of the method of the invention is that energy-intensive alternate cooling and heating of the CO<sub>2</sub> is avoided.

35 CO<sub>2</sub> to be fed in is taken from a liquid CO<sub>2</sub> reservoir and cooled by means of a heat exchanger. The CO<sub>2</sub> conveyed by the pump is brought to the separation temperature by means of the heat exchanger. However, this can be very small since the CO<sub>2</sub> is only heated from typically 0-5°C to

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5-20°C.

5 If low separation temperatures, e.g. 5°C, are chosen, this heat exchanger can also be taken out of operation. The liquid CO<sub>2</sub> flows through the separation column. By means of a switching valve, the individual fractions are diverted to fractionation columns, for example to three fractionation columns. Any number of fractionation columns are possible, but in the simplest case there is one fractionation column and one additional pipe which connects the switching valve to a further switching valve.

10

The column outward pressure is regulated by means of a pressure regulating valve. At this valve, the CO<sub>2</sub> is depressurized. After injection of the sample (assumption: the sample contains 3 components A, B and C), the sample is separated on the separation column.

15

During elution of component A, the switching valve is set so that CO<sub>2</sub> and component A is diverted in the direction of the further switching valve. The liquid CO<sub>2</sub> is depressurized. The CO<sub>2</sub> loses its solvent power. Component A is thus retained on the fractionation column. The CO<sub>2</sub> is cooled further (if necessary) by means of a heat exchanger and goes back to the reservoir.

20

After elution of component A from the column, the switching valve is switched over to a further fractionation column. The component B to be eluted is retained on this fractionation column. Component C is retained on a further fractionation column. When no sample substance is eluted, the CO<sub>2</sub> stream can be conveyed either via one of the fractionation columns or via an additional pipe which is provided with a pressure regulating valve and connects the switching valve to the further switching valve.

30

The chromatographic material of the separation column is chosen so that a maximum resolution of the sample components is achieved. The material of the fractionation columns is chosen so that maximum adsorption of the respective sample component is achieved under the conditions prevailing there during the separation.

35

This procedure can be repeated for a plurality of injections (until the capacity of a fractionation column has been reached). Elution of the

respective components is carried out using a suitable strong solvent, e.g. ethanol. A separate pump conveys the eluant via the switching valve to the outlet downstream of the further switching valve. The desired fractions are thus collected as solutions in liquid in this case.

5

The advantage of this apparatus described is that neither a fourth heat exchanger nor a precipitator is required.

10

In further embodiments of the method of the invention, the pressure regulating valve can be installed directly downstream of the separation column. The expanded CO<sub>2</sub> together with the respective sample component is conveyed only after depressurization by means of the switching valve to the appropriate fractionation column. The advantage over the above-described arrangement is that only one pressure regulating valve is required.

15

In further embodiments of the method of the invention, the pressure regulating valves can be installed downstream of the fractionation columns. In addition, further precipitators are located downstream thereof.

20

In this arrangement, the pressure is regulated downstream of the fractionation column. The material of the fractionation columns is selected so that the components A, B and C are retained in the appropriate fractionation column under the prevailing conditions. After repeated injection and collection of the sample components on the fractionation columns, the individual components can be eluted by increasing the elution strength of the CO<sub>2</sub> and/or adding a modifier and be separated from the CO<sub>2</sub> by means of the respective precipitator.

25

As an alternative, elution can also be carried out using a liquid eluant. In this case, the precipitators can be omitted. The apparatus can also be modified by using only one precipitator downstream of the switching valve instead of three precipitators.

30

A customary SFC unit can be operated much more efficiently in energy terms by means of the method of the invention. In the heat exchanger downstream of the reservoir, the CO<sub>2</sub> has to be heated to only a small degree, if at all. In addition, the lower pressure upstream of the pressure

35

regulating unit results in reduced cooling due to the adiabatic expansion and much less energy therefore has to be supplied to the heat exchanger downstream of the separation column. In principle, the method of the invention can be applied to any SFC unit.

5

The method of the invention is very particularly useful for the column-chromatographic separation of unsaturated, in particular polyunsaturated, higher fatty acids or derivatives thereof, e.g. esters or lipids, preferably from natural oils such as vegetable oil, alga oil, oil from microorganisms or fungi and also fish oil, and thus for the isolation and purification of SDA (stearidonic acid, 18:4, n-3), AA (arachidonic acid, 20:4, n-6), ETA (eicosatetraenoic acid, 20:4, n-3), EPA 25 (eicosapentaenoic acid, 20:5, n-3), DPA (docosapentaenoic acid, 22:5, n-3), DHA (docosahexaenoic acid, 22:6, n-3), GLA (gamma-linolenic acid, 18:3, n-6), ALA (alpha-linolenic acid, 18:3, n-3), DPA (docosapentaenoic acid, 22:5, n-6).

The following examples serve to illustrate the invention without restricting it to these examples.

20 Example 1:

A customary SFC apparatus as described above was used. The columns used had a length of up to 170 cm. The working pressures normally employed were generally from 120 to 350 bar. These columns can be used in SFC since the pressure drops over a column of this length are only small (normally 20-50 bar). The low pressure drops make it possible to use higher flows which in SFC, in contrast to HPLC, lead to only a small decrease in efficiency. Carbon dioxide was used as eluant. The operating parameters are listed in table 1 below. In addition to the use according to the invention of liquid or fluid carbon dioxide, supercritical carbon dioxide was also used.



Table 1: Operating parameters

Calculation p	Column: 101	L(cm)	d(cm)	r(cm)	dp( $\mu$ m)	Type
		110	9	4.5	25	
	$\rho$ (g/ml)	$\eta$ dyn.(Pa s)	$\eta$ dyn.(cpoise)	F(kg/h)	F(ml/min)	$\Delta p$ (bar)
80 bar, 50°C	0.219	2.05E-05	0.02053	300	22 831.05	21.6 SFC
100 bar, 50°C,	0.3899	2.95E-05	0.02945	300	12 823.80	17.4 SFC
200 bar, 50°C	0.784	6.80E-05	0.06797	300	6 377.55	20.0 SFC
25°C, 150 bar	0.8771	8.63E-05	0.0863	300	5 700.60	22.7 SbFC
0°C, 150 bar	1	1.23E-04	0.1234	300	5 000.00	28.4 SbFC
0°C, 300 bar	1.055	1.47E-04	0.1466	300	4 739.34	32.0 SbFC
0°C, 40 bar	0.9333	1.01E-04	0.1009	300	5 357.33	24.9 LFC
10°C, 50 bar	0.8705	8.00E-05	0.08	300	5 743.83	21.2 LFC
25°C, 70 bar	0.744	6.04E-05	0.06043	300	6 720.43	18.7 LFC
MeOH, 20°C	0.79	5.84E-04	0.584	300	6 329.11	170.4 HPLC
EtOH, 20°C	0.789	1.20E-03	1.201	300	6 337.14	350.9 HPLC

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Calculation according to:  $\Delta p = \frac{F \cdot \eta \cdot L \cdot 1000}{60 \cdot \pi \cdot r^2 \cdot dp^2}$

where

- |    |            |   |
|----|------------|---|
| 5  | $\Delta p$ | pressure drop in [bar]                      |
|    | F          | volume flow of the eluant in [ml/min]       |
|    | L          | length of the column in [cm]                |
|    | $\eta$     | viscosity in [cpoise]; 1000 cpoise = 1 Pa s |
|    | r          | internal radius of the column in [cm]       |
| 10 | Dp         | particle diameter in [ $\mu\text{m}$ ]      |

Example 2:

Separations of an ethyl ester of fish oil on two different stationary phases were carried out. In each case, both supercritical conditions and also a  
5 temperature/flow combination in the range according to the invention were chosen. In table 2 below, the purities of the EPA and/or DHA of the fractions collected are compared with one another.

A fish oil was separated under various conditions: liquid phase ( $T = 18^{\circ}\text{C}$ ,  
10  $p_n = 52 \text{ bar}$ ;  $F = 300 \text{ kg/h}$ ); supercritical phase: ( $T = 46^{\circ}\text{C}$ ,  $p_n = 105 \text{ bar}$ ;  $F = 300 \text{ kg/h}$ ); stationary phase: aminopropyl; mobile phase:  $\text{CO}_2$ .

The EPA content in % of the respective fraction is shown in table 2. It can  
15 easily be seen that the profile is very similar. However, the separation in the liquid range according to the invention was much quicker.

A reduction from about 75 min to only 40 min was possible. The absolute  
purity which could be achieved was somewhat lower (about 90% in the liquid range instead of 95% under supercritical separation conditions), but  
20 if the yield calculation is based on the fractions having an EPA purity above 80%, this corresponded to 52.7% of the mass injected in the supercritical range and 52.0% in the liquid range. These figures make it clear that the separations achieved are very similar.

25 However, the plants necessary for the liquid range are significantly cheaper since they only have to be suitable for pressures up to about 70 bar and do not have to be designed for up to 300 bar.

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Table 2: EPA content in % in the case of separation using liquid carbon dioxide      EPA content in % in the case of separation using supercritical carbon dioxide

Separation time (min)	EPA content of the fraction (%)	Separation time (min)	EPA content of the fraction (%)
12	1.4	21	2.03
12.5	0.18	23	0.99
13	0.12	25	0.29
13.5	0.25	28	0.54
14	0.19	30	3.01
14.5	0.23	32	13
15	0.53	33	14.23
15.5	3.61	34	14.35
16	13.94	35	18.63
16.5	27.28	36	25.5
17	44.65	37	36.16
17.5	64.79	38	54.23
18	73.59	39	76.62
18.5	79.96	40	91.08
19	84.14	41	93.96
19.5	86.69	42	94.78
20.5	89.07	43	95.18
21	89.64	44	95.44
21.5	89.45	45	95.31
22	88.05	46	95.25
22.5	87.01	47	94.34
23	85.76	48	93.67
23.5	84.1	49	93.03
24	82.16	50	92.34
24.5	80.53	51	91.7
25	78.8	52	91.24
25.5	76.97	53	90.67
26	75.63	54	89.42
26.5	74.09	56	86.79
27	73.21	59	79.34
28.5	69.27	62	61.96
32	59.55	67	14.71
34	51.7	121	1.95
37	26.52		
40	17.67		

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In table 3, the purities of the fractions in respect of both EPA and DHA are listed for the same separations. Here too, it can be seen that the separations are comparable, although the separation in the liquid range can be carried out in half the time using this phase.

5

A fish oil was separated under various conditions: liquid phase (T = 18°C, pn = 52 bar; F = 300 kg/h); supercritical phase: (T = 46°C, pn = 105 bar; F = 300 kg/h); phase: aminopropyl; mobile phase: CO<sub>2</sub>

Table 3: EPA/DPA content in % in the case of separation using liquid carbon dioxide		EPA/DPA content in % in the case of separation using supercritical carbon dioxide	
Separation time (min)	EPA/DPA content of the fraction (%)	Separation time (min)	EPA/DPA content of the fraction (%)
12	1.4/2.99	21	2.03/85.53
12.5	0.18/0.31	23	0.99/9.92
14	0.12/0.18	25	0.29/0.65
13.5	0.25/0.32	28	0.54/0.27
14	0.19/0.26	30	3.01/0.37
14.5	0.23/0.27	32	13/0.28
15	0.53/0.12	33	14.23/02
15.5	3.61/0.16	34	14.35/0.01
16	13.94/0.01	35	18.63/0.01
16.5	27.28/0.11	36	25.5/0.15
17	44.65/0.01	37	36.16/0.01
17.5	64.79/0.01	38	54.23/0.01
18	73.59/0.01	39	76.62/0.22
18.5	79.96/0.01	40	91.08/0.01
19	84.14/0.01	41	93.96/0.01
19.5	86.69/0.01	42	94.78/0.01
20.5	89.07/0.32	43	95.18/0.13
21	89.64/0.75	44	95.44/0.01
21.5	89.45/1.41	45	95.31/0.27
22	88.05/2.58	46	95.25/0.35
22.5	87.01/3.72	47	94.34/0.44
23	85.76/5.15	48	93.67/0.58
23.5	84.1/6.66	49	93.03/0.72

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24	82.16/8.14	50	92.34/0.93
24.5	80.53/10	51	91.7/1.2
25	78.8/11.66	52	91.24/1.48
25.5	76.97/13.17	53	90.67/1.88
26	75.63/14.81	54	89.42/2.43
26.5	74.09/16.12	56	86.79/3.65
27	73.21/17.31	59	79.34/8.53
28.5	69.27/21.06	62	61.96/21.54
32	59.55/28.9	67	14.71/62.52
34	51.7/35.27	121	1.95/81.27
37	26.52/54.38		
40	17.67/62.17		

- Tables 4 and 5 show the results of a separation of the same starting material on a different stationary phase. Here too, it can be seen that the separations under the two conditions are very similar. On this stationary phase, however, the substances elute later under liquid conditions. The maximum EPA purity which can be achieved on this stationary phase is about 80%. If, for comparison, an EPA purity of more than 70% is defined for the mass yield, the yield of 36.2% under liquid conditions is significantly higher than in the case of supercritical conditions where it is only 30.3%.
- 10 The strongly pronounced tailing of both of the EPA and the DHA is presumably attributable to silanol groups which are still highly active. This phase is less suitable for the separation. However, it was able to be shown that the separations in the various states are comparable.
- 15 A fish oil was separated under different conditions: liquid phase ( $T = 18^{\circ}\text{C}$ ,  $p_n = 52$  bar;  $F = 300$  kg/h); supercritical phase: ( $T = 46^{\circ}\text{C}$ ,  $p_n = 160$  bar;  $F = 300$  kg/h); stationary phase: silica gel; mobile phase:  $\text{CO}_2$ .

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Table 4: EPA content in % in the case of separation using liquid carbon dioxide      EPA content in % in the case of separation using supercritical carbon dioxide

Separation time (min)	EPA content of the fraction (%)	Separation time (min)	EPA content of the fraction (%)
20	13.02	12	9.11
21	20.05	13	4.15
23	34.82	14	10.06
24	47.25	15	21.46
25	62.5	15.5	29.13
26	71.44	16	40.14
26.5	74.5	16.5	54.65
27	76.22	17	63.67
27.5	77.39	17.5	68.77
28	78.59	18	73.71
29	79.85	19	78.4
30	80.05	19.5	80.8
31	79.39	20	81.48
32	76.37	21	80.31
33	74	22	76.77
34	70.8	23	72.49
35	68.48	24	69.63
36	66.17	25	66.77
37	64.6	26	64.32
38	64	27	66.69
39	62.62	28	60.73
40	61.82	29	59.48
41	60.99	30	58.03
42	60.62	32	54.65
44	59.23	34	51.4
46	57.65	36	46.5
48	56.66	38	42.34
50	55.32	40	37.86
52	53.51	45	27.81
55	48.97		
60	44.15		

EPA/DPA content in % in the case of separation using liquid carbon dioxide		EPA/DPA content in % in the case of separation using supercritical carbon dioxide	
Separation time (min)	EPA/DHA content of the fraction (%)	Separation time (min)	EPA/DHA content of the fraction (%)
20	13.02/1.69	12	9.11/26.52
21	20.05/0.92	13	4.15/3.59
23	34.82/0.48	14	10.06/1.12
24	47.25/0.65	15	21.46/0.45
25	62.5/0.92	15.5	29.13/0.41
26	71.44/1.38	16	40.14/0.39
26.5	74.5/1.63	16.5	54.65/0.52
27	76.22/1.93	17	63.67/0.73
27.5	77.39/2.19	17.5	68.77/0.97
28	78.59/2.54	18	73.71/1.43
29	79.85/3.42	19	78.4/2.23
30	80.05/4.59	19.5	80.8/2.68
31	79.39/6.21	20	81.48/3.21
32	76.37/8.36	21	80.31/5.23
33	74./1.88	22	76.77/8.96
34	70.8/15.08	23	72.49/13.52
35	68.48/17.73	24	69.63/16.38
36	66.17/19.53	25	66.77/19.32
37	64.6/21.21	26	64.32/22.11
38	64/22.2	27	66.69/20.21
39	62.62/23.39	28	60.73/25.81
40	61.82/24.35	29	59.48/27.33
41	60.99/24.8	30	58.03/28.62
42	60.62/25.12	32	54.65/31.5
44	59.23/26.89	34	51.4/34.19
46	57.65/28.31	36	46.5/38.47
48	56.66/29.39	38	42.34/42.23
50	55.32/30.58	40	37.86/45.76
52	53.51/31.95	45	27.81/53.76
55	48.97/34.7		
60	44.15/38.6		



	HPLC	high performance liquid chromatography
	SFC	chromatography using supercritical gas
5	SbFC	chromatography using fluid gas ( $T < T_{kr}$ ; $p > p_{kr}$ )
	LFC	chromatography using liquefied gas ( $T < T_{kr}$ ; $p < p_{kr}$ )
	EPA	ethyl eicosapentaenoate (20:5)
	DHA	docosahexaenoic acid (22:6)

Claims

1. A method for the column-chromatographic separation of mixtures of materials using a fluid or a mixture of fluids which are gaseous at 25°C and 1 bar in a liquid, nonsubcritical and noncritical state as eluant.  
5
2. The method as claimed in claim 1, characterized in that the eluant is selected from the group consisting of liquid dinitrogen oxide, liquid fluorinated hydrocarbons and chlorofluorocarbons, liquid carbon dioxide, liquid sulfur hexafluoride, liquid propene, liquid propane,  
10 liquid ammonia, liquid sulfur dioxide, liquid xenon, liquid ethane and mixtures thereof.
3. The method as claimed in claim 1, characterized in that the eluant has a dynamic viscosity of  $10^{-4}$  -  $10^{-5}$  Pa s, preferably  $2 \cdot 10^{-4}$  -  $2 \cdot 10^{-5}$  Pa s, a density of 0.5 - 1.2 g/ml, preferably 0.5 - 1.2 g/ml.  
15
4. The method as claimed in claim 1, characterized in that the eluant is liquid carbon dioxide, preferably in a temperature range from 0 to 20°C.  
20
5. The method as claimed in claim 1, characterized in that the eluant is liquid carbon dioxide in a pressure range from 30 to 150 bar.
6. The method as claimed in claim 1, characterized in that the eluant comprises at least one modifier.  
25
7. The method as claimed in claim 1, characterized in that a column length of at least 10 cm is used.
8. The method as claimed in claim 7, characterized in that a column length of from 0.25 to 2.0 m is used.  
30
9. The method as claimed in claim 8, characterized in that a column length of from 1.1 to 1.7 m is used.  
35
10. The method as claimed in claim 1, characterized in that the stationary phase used is a modified or unmodified phase selected

from the group consisting of silica gel, aluminium oxide, titanium dioxide and supported polymeric stationary phases.

- 5 11. The method as claimed in claim 10, characterized in that the stationary modified phase is selected from the group consisting of a diol phase, aminopropyl phase, RP8 and RP18 phase applied to silica gel, aluminum oxide or titanium dioxide.
- 10 12. The method as claimed in claim 1, characterized in that the mixtures of materials used are natural oils comprising unsaturated fatty acids.
- 15 13. The use of fluids or a mixture of fluids which are gaseous at 25°C and 1 bar in the liquid, nonsubcritical and noncritical state for the isolation and/or purification and/or preparative recovery of mixtures of materials by means of column chromatography.

## Abstract

### Column-chromatographic separation method

- 5 A description is given of a column-chromatographic separation method of the liquid chromatography type by means of eluants as mobile phase comprising lower molecules, preferably carbon dioxide, for the isolation and/or purification and/or preparative recovery of mixtures of (natural) materials.

**COMBINED DECLARATION AND POWER OF ATTORNEY**

Attorney Docket No.

199kd02.us (9600\*2)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "Column-Chromatographic Separation Method" the specification of which

(check one) ☐ is attached hereto.☒ was filed on January 18, 2002 asApplication Serial No. 10/069,080 andwas amended on \_\_\_\_\_  
(if applicable)was amended through \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or (f) or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's or plant breeder's rights certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

**Prior Foreign Application(s)****Priority Claimed**

<u>199 34 168.0</u> (Number)	<u>Germany</u> (Country)	<u>21 July 1999</u> (Day/Month/Year Filed)	<input checked="" type="checkbox"/> <input type="checkbox"/> Yes No
<u>PCT/EP00/06853</u> (Number)	<u>International</u> (Country)	<u>18 July 2000</u> (Day/Month/Year Filed)	<input checked="" type="checkbox"/> <input type="checkbox"/> Yes No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/> <input type="checkbox"/> Yes No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status)
_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)

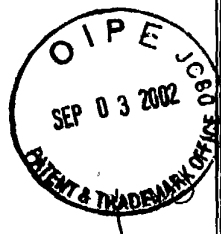
I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

_____ (Application No.)	_____ (Filing Date)
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

3.0005.00150 0040342

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) associated with CUSTOMER NUMBER 23416; all of CONNOLLY BOVE LODGE & HUTZ LLP, as attorneys with full power of substitution to prosecute this application and conduct all business in the Patent and Trademark Office connected therewith.



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